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DIPLOIDIZATION IN FLAX RUST

by

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A THESIS

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The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies for
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ABSTRACT

The introduction, simultaneously, of haploid pycnial and diploid uredinial stages of Melampsora lini to flax leaves, resulted in the production of diploid aecia. Since aecial formation resulting from normal fusions of pycnial elements had been precluded, and since check leaves inoculated with only one stage did not produce aecia, it was concluded that diploidization of the haploid pycnial stage by the diploid uredinial stage had occurred. Because occasionally aecia appeared in the presence of only rudimentary pycnia, or even when no pycnia were discernible, it was postulated that diploidization of pycnial elements could occur in stages as early as the basidiospore germ tube.

The capacity of the diploid uredinial stage of flax rust to diploidize the haploid pycnial stage may be utilized advantageously in flax rust research. Besides permitting a reduction of the time and material presently required, it also makes available a more efficient method for detecting rust races whose virulence is masked by heterozygosity.

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INTRODUCTION

Studies of rusts that necessitate their reproduction are complicated by several factors. In the normal procedure used to identify unknown flax rust races, i.e. inoculation of a series of host differentials with an unknown race of rust, the heterozygotes will tend to escape observation (20). This is because the genes for virulence in rust are recessive, and a race heterozygous at a particular locus would not infect a specific differential host. This means that a potentially virulent race of rust could remain undetected, which, of course, is a considerable hazard when breeding for rust resistance. Also, because rusts are obligate parasites, and because many species are heteroecious (7, 28), the simultaneous production of several host populations is often required. In addition, the complicated life cycle of the rusts makes it necessary to grow them through different stages. Because of the difficulties introduced by these factors, any means of minimizing or overcoming them would greatly benefit the researcher or breeder.

The work of several investigators have contributed greatly to the understanding of the behavior of rusts, and thereby have pointed the way to more efficient means of conducting future investigations.

Perhaps one of the most important of these contributions was the discovery by Craigie of the sexual stages in the life cycles of Puccinia graminis (Pers.) (wheat stem rust) and P. helianthi (Schw.) (sunflower rust) (14, 15). His observation, that pycniospores of one type fertilized pycnial flexuous hyphae of an opposite type, established

that the pycnial stage was also the sexual stage. Subsequently he observed the same situation in other rust species (16) which indicated that the sexual stages in most rusts were probably similar. As a result of these observations a method of making controlled crosses was revealed.

A second contribution was the demonstration by Brown (8) that in P. helianthi the fusion of two haploid hyphae could produce a diploid stage viz. the coalescence of two pycnia led to the formation of an aecium. He also observed that sunflower leaves bearing pycnia when inoculated with urediniospores would produce aecia (diploidization of a haploid mycelium by a diploid mycelium). The importance of this phenomenon in rusts was shown by Garrett and Wilcoxson's (21) demonstration that uredinial pycnial diploidization also occurred in P. graminis. Cotter (13) extended this to show that diploidization in P. graminis was possible when either urediniospores or aeciospores were used in conjunction with pycnia. However, since aeciospores develop into the uredinial stage, it is very probable that this diploidization with aeciospores was essentially the same as that induced with urediniospores.

The phenomenon of diploidization of a haploid stage by a diploid stage was given the name "Buller phenomenon" by Quintanilha, and is defined by Buller (11) as: " . . . the diploidization of a unisexual mycelium or unisexual rudiment of a fructification by a bisexual mycelium," with the qualification: " . . . if we recognize that a dikaryotic diploid mycelium of a . . . rust fungus is just as bisexual as a bisexual mycelium of . . . Neurospora." The use of this method of diploidization offers several advantages to the investigator. For example, in making crosses between rust races, one race may be maintained as urediniospores. This eliminates several stages, which

in the case of dioecious rusts, also means a reduction of the alternate host population. The need to break the dormancy of the teliospores is also eliminated, thus avoiding what at times is a rather cumbersome procedure.

As has been mentioned previously, the occurrence of a particular phenomenon in one or two species of rust indicates that the occurrence of a similar phenomenon in other species is quite probable. It is therefore reasonable to expect that diploidization could be induced in species other than P. graminis and P. helianthi. The advantages to be gained by the use of diploidization in a species of rust as economically important as Melampsora lini (Pers.) Lev. (flax rust) would be of inestimable value. Although it has been known for some time that the phenomenon of diploidization occurs in P. graminis and P. helianthi, its practical application has been somewhat limited to date. It is possible that certain technical problems would have to be solved before the potential advantages of the use of diploidization may be realized.

LITERATURE REVIEW

The classification of flax rust, as given by Wolfe and Wolfe (27) is: class Basidiomycetes; order Uredinales; family Melampsoraceae; genus Melampsora; species Melampsora lini (Pers.) Lev. It is an autoecious, obligate parasite of flax, Linum usitatissimum, and has a life cycle typical of many Uredinales in that it includes five spore forms; teliospores, basidiospores, pycniospores, aeciospores and urediniospores. The basidiospores and pycniospores are haploid, the aeciospores and urediniospores are binucleate (dicaryotic), and the teliospores are dicaryotic at first, but a subsequent fusing of the nuclei causes them to become uninucleate and diploid. Meiosis occurs either in a germinating teliospore or in the resulting basidium, and produces four haploid basidiospores. Each basidiospore is capable of reinfecting the host plant and producing a pycnium (Allen, 4).

Craigie (14, 15) discovered the sexual stage in wheat stem rust, Puccinia graminis, and sunflower rust, P. helianthi, when he observed insects feeding on pycnial nectar, and conjectured that they transported pycniospores from one pycnium to another, thereby inducing fertilization. He demonstrated the feasibility of his conjecture by establishing that an aecium would not form when a pycnium was protected from possible contact with pycniospores from other pycnia. He later showed, in P. graminis, P. coronata Cda. (crown rust of oats) and P. pringsheimiana Kleb. (gooseberry rust) (16, 17) that of the four basidiospores produced by a basidium, two were of one phase (plus strain), and two were of another phase (minus strain), and that fertilization occurred between pycnia only when they were produced by basidiospores of opposite phase.

Andrus (5), from the results of his work with Uromyces appendiculatus Pers., concluded that fertilization was initiated when a pycniospore of one type fused with a receptive hypha in a pycnium of another type. He believed the pycniospore nucleus then migrated down the receptive hypha to the protoaecium (aecial basal cells), which he called "egg cells," and there associated with an "egg cell" nucleus, after which the nuclei went into a series of conjugate divisions and the resulting cells formed an aecium. Hanna (22), however, on the basis of the results obtained with P. graminis, postulated that a pycniospore of one type, on contacting a pycnium of another type, produced a germ tube which grew down through the pycnium, and on reaching the protoaecium, fused with one of the basal cells, thus making conjugate association of the two types of nuclei possible. Both Andrus and Hanna agree that conjugate association of the two types of nuclei is a preliminary step to aecial formation, and that this association occurs in the protoaecium, but they differ in opinion as to the manner in which the pycniospore nucleus arrives at the point of association. Buller (10), Craigie (17) and Pierson (26) on the basis of independent observations on P. graminis, P. helianthi and Cronartium ribicola Dietr. respectively, agree that conjugate association of the two types of nuclei occurs in the protoaecium, but they believe that the pycniospore fuses directly with a receptive hypha and produces no germ tube. In a more recent work, Craigie and Green (18) studied the behavior of fertilizing pycniospore nuclei of P. graminis and concluded that, typically, fusion occurs between pycniospores and receptive hyphae of opposite mating types, and that the pycniospore nucleus, while undergoing a series of divisions, migrates down to the protoaecium where conjugate association begins. Allen, however, after studying pycnial

fertilization in P. graminis and P. coronata Cda. (1, 2, 3), contends that conjugate association of the two types of nuclei occurs before the pycniospore nucleus reaches the protoaecium. She believes that the "sporophytic generation" might originate by fusion of pycniospore germ tubes of opposite mating types, or by fusion of a fertilizing pycniospore germ tube with any of the other elements of the receptive pycnium, such as paraphyses, mycelia, or wall cell. From the point of fusion, "sporophytic hyphae" would spread and eventually invade the aecial primordium where they would give rise to, or participate in producing, aeciospores. She subsequently made a study of the behavior of fertilizing pycniospore nuclei of M. lini (4) in which her observations supported those she had made in P. graminis and P. coronata.

Although the above-mentioned works do not agree on the method or site of initial conjugate association, they do establish that the principle of fertilization of pycnia by pycniospores of different mating type is common to several different species of rusts. Olive (24) reviewed most of the works mentioned here and many others on heterothallism in different classes of fungi, and concluded that heterothallism is perhaps a common feature of most Uredinales. He suggested that even some of the reported exceptions, after more thorough investigation, might also prove to be heterothallic.

Brown (8) took sunflower leaves bearing pycnia of P. helianthi and infected them with urediniospores. He noted that aecia were produced although apparently no pycnial interfertilization had occurred. He concluded, therefore that uredinial and pycnial hyphae had coalesced, and that nuclei of opposite mating types from each had associated, and, after conjugate division, had produced aecia. Garrett and Wilcoxson (21)

achieved similar results in P. graminis. They observed that, after fusion of uredinial germ tubes with pycnial hyphae, aecia were ultimately produced. Cotter (13) demonstrated that, in P. graminis, aecia were also formed when barberry leaves (Berberis vulgaris) on which pycnia were present were inoculated with either urediniospores or aeciospores.

Buller reviewed the many reported instances of diploidization in plants, animals and fungi (11), and concluded that diploidization is essentially a sexual process. He considered that diploidization of a haploid mycelium by a diploid mycelium was, in effect, a sexual union between unisexual (haploid) and bisexual (diploid) elements (Buller Phenomenon). He also stated that he introduced the term "diploidization" in 1930 and defined it as "the process by which a haploid cell is converted to a diploid cell, or a haploid mycelium into a diploid mycelium by the formation of a pair or pairs of conjugate nuclei within the cell or within the mycelium."

In certain investigations, e.g. the present one, the ability to maintain leaves or other portions of a plant in healthy state for a prolonged period of time permits greater experimental control than would otherwise be possible. The use of different chemicals for this purpose has been investigated by several workers.

Person et al. (25) demonstrated that detached wheat leaves floated on benzimidazole solution of from 30 to 100 parts per million, when compared with leaves floated on distilled water, retained more of their chlorophyll content and had a reduced respiration rate. They also noted that the soluble nitrogen and amino acid content increased in the distilled water but not in the benzimidazole solution, and that

the insoluble nitrogen to dry weight ratio of the leaves floated on benzimidazole solution remained fairly constant. This would indicate a more stabilized nitrogen metabolism and a higher protein conservation in the leaves floated on benzimidazole solution. They also found that floating the leaves on a 5 p.p.m. kinetin (6-furfuraminopurine) solution had similar advantageous effects.

Richmond (27) observed that detached xanthium leaves kept in water lost most of their chlorophyll in four or five days, and 60% of their protein nitrogen in twelve days. But when leaves were kept in a 1 p.p.m. kinetin solution their chlorophyll loss was greatly reduced and their protein nitrogen dropped only 50%. In a 5 p.p.m. kinetin solution, leaves remained green even after twenty days, and the protein nitrogen loss was only 15% after twelve days.

Dedolph et al. (19) tested the effects of benzimidazole, kinetin and an analogue of kinetin, N⁶ - benzylaminopurine, on the retention of color and general appearance of broccoli in storage. They compared the effects, after prolonged storage at different temperatures, of post-harvest dipping the plants in 10 p.p.m. of each of the chemicals. They concluded that kinetin generally caused the broccoli to maintain a more satisfactorily marketable appearance; the N⁶ - benzylaminopurine caused a greater retention of chlorophyll; the benzimidazole solution had no apparent effect.

MATERIALS AND METHODS

The variety of flax "Bison" was selected for use in this study because of its general susceptibility to the known races of M. lini. Flax stems bearing teliospore sori were gathered from the stubble of plants grown in variety trials the previous summer. The stems were collected at different times during the winter, as required. No attempt was made to select particular races or to identify them.

The variety "Redwing," grown in the greenhouse, was used in secondary trials, both as a host and as a source of teliospore sori.

Several methods were tried in an attempt to break teliospore dormancy. The most successful was one devised by Clark (12) (from a postdoctorate thesis by K. W. Sheppard), which consisted of the following sequence of treatments:

The teliospores were dried and placed in a temperature of approximately -5° C for two days; they were then removed, moistened, and incubated for two days at 13° C. These steps were repeated for twenty days, i.e. five times. The teliospores were then incubated for four to five days at 13° C, or stored at 5° C for future use.

To preclude any possibility of pycnial interfertilization occurring, an attempt was made to obtain leaves bearing only single pycnia. One method adopted to achieve this was to inoculate individual leaves with single basidiospores. Another method was to suspend germinating teliospores over flax plants, permitting broadcast inoculation by basidiospores and select those leaves on which only a single pycnium appeared.

Because basidiospores are relatively small and hyaline and therefore difficult to see, it was necessary, in the single-spore inoculation method, to keep them under as high a magnification as possible during transfer from the basidium to the leaf. It was advantageous therefore to detach the leaves from the plant in order to place them under a microscope. Because pycnia are rather inconspicuous, when it came to selecting those leaves which bore only a single pycnium, considerable magnification was also required, which again made it advantageous to detach the leaves from the plants. To maintain these detached leaves in a healthy condition a suitable medium was needed. The use of benzimidazole and kinetin (19, 25, 27) was primarily considered for this purpose, although indole acetic acid and two analogues of kinetin, 6 anilinopurine and 6 benzylaminopurine were also tested. Flax leaves were floated in 0 (distilled water), 1, 5, 10, 20, 30 and 40 parts per million solutions of each of the compounds. The treatment which appeared to keep the leaves most healthy for the longest period of time was selected (in this case the 10 p.p.m. kinetin solution) and used throughout the trials, wherever single leaf flotation was required. Although the concentrations over 10 p.p.m. of kinetin and the two analogues were also relatively effective in maintaining the leaves in a healthy condition, they appeared to reduce the germination rate of basidiospores.

The descriptions and illustrations provided by Allen (4) and Arthur (6) furnished the basis for establishing a practical method of readily identifying the different stages of flax rust. They were adequate for identifying the telial, basidial and pycnial stages since the appearance of each of these stages was sufficiently unique to prevent it being mistaken for any other stage. The aecial and uredinial

stages, however, have several similarities, which made it necessary to ascertain what the most salient features of each were. To this end, several flax leaves were inoculated with basidiospores and the resultant stages of infection were progressively examined, superficially and in section. The features which most consistently served to distinguish each stage were observed and are indicated in the photographs which were taken of the results of the trials.

The most distinguishing feature of uredinia was found to be the presence of sterile hyphae or paraphyses, which are readily identified by their flask-like shape, smooth surface and lack of pigment (figs. 1 and 2). They appear early in the development of the uredinia and persist until the pustule disintegrates, but are never present in aecia.

Another means of distinguishing uredinia from aecia was found to be the manner in which the spores are borne. Urediniospores originate from a compact cell mass at the base of the uredinium and are pedicellate. (fig. 1). Aeciospores originate from elongated, forked, basal cells and are borne in chains, in which they are separated from each other by short-lived intercalary cells. The aecial basal cells tend to flare out in a fan-like fashion towards the periphery of the aecium (figs. 13 and 14), and to maintain this position until the aecium opens. When the aecium opens, the pressure on the aeciospores and the basal cells is released, and this permits the basal cells to assume a more upright position (fig. 16). Aecial basal cells persist throughout aecial development, whereas the uredinial cell mass decreases in size progressively as the cells differentiate into urediniospores (figs. 1 and 2).

Because the compactness of aeciospores within an aecium tends to obscure the presence of intercalary cells, and because these cells

tend to disintegrate early, the typical chain-like appearance of aeciospores, as depicted by Allen (4), is not normally apparent. However, since intercalary cells occur only between aeciospores, and since a certain proportion of them is usually detectible, their presence does serve as a useful feature in differentiating aecia from uredinia. The physical differences between aeciospores and urediniospores were of little practical value as differentiating features because, besides being very similar in shape, size, color and texture, both types of spores normally suffered considerable distortion from being closely compressed within the pustules (figs. 1, 8, 13 and 16). Other characteristics of uredinia and aecia, such as the shape, size or location of the pustule, were found to be either too variable or too indefinite, and were therefore also of little value for differentiating between the two stages.

Pycnia revealed their presence, on maturity, by exuding a drop of nectar on to the leaf surface. However, since it was desirable to detect them before sporulation occurred, it was necessary to discover a method of recognizing pycnia at an earlier stage. It was found that under slight magnification a developing pycnium could be detected by the appearance of a small, clear, membranous spot on the leaf surface. Although aecia and uredinia also initially formed similar spots, the spots they formed rapidly enlarged and soon took on a yellow cast when the underlying spores began to develop. The spots formed by pycnia enlarged very little, however, and remained colorless. In section, immature pycnia were detected quite readily by their pyramidal shape and by the early appearance of spores and elongated cells which developed into trichogynes (figs. 7 and 8). The spores were a

particularly distinctive feature because of their relatively small size and because of the discovery that they take up safranin or fast green readily.

To inoculate the leaves, three different methods were used:

(1) Single spore inoculations - A single basidiospore was drawn into a fine glass tube and deposited in a droplet of water on each leaf. Individual leaves were then floated on a 10 p.p.m. kinetin solution of pH 7. The cultures were kept in a moist chamber at 13° C for twenty-four hours. They were then brought to room temperature, at which they were maintained while periodic examinations for the appearance of pycnia were made. Whenever a pycnium appeared, urediniospores were brushed onto the surrounding area of the leaf. The leaves thus treated were then placed in a temperature of 5° C for approximately one hour and then returned to room temperature. This induced the formation of a fine layer of condensation over the leaf surfaces. The leaves were kept under a cover to retard evaporation, placed in a temperature of 13° C for twenty-four hours, and then returned to room temperature and inspected periodically for the appearance of aecia.

Twenty single-basidiospore inoculations were made and pycnia were detected on eight of the leaves. Four of these were brushed with urediniospores, four were maintained as controls.

(2) Spore suspension method - Moisture was induced to form on several leaves, as in method 1. Over these leaves moistened pieces of flax stems bearing germinating teliospores were suspended for up to twelve hours at 13° C (the duration of suspension depended on the rate of basidiospore production). The teliospores were then removed and the leaves retained at 13° C for a total period of twenty-four hours, after which they were kept at room temperature and inspected periodically for the appearance of pycnia. Further treatment of selected leaves was performed as in method 1.

The use of this method tended to introduce heavy infections by other organisms so that the detection of pycnia proved rather difficult. The teliospore sori were therefore washed in running water for up to twenty-four hours in an attempt to remove the spores of these other organisms. This was accomplished by placing the stem portions bearing the sori in a funnel and connecting the funnel to a water tap with a rubber tube. The wide end of the funnel was covered with a fiberglass screen which served as a trap for the stem portions. The water was then permitted to flow up into the funnel and overflow through the fiberglass screen. Although this did not eliminate other organisms entirely, it did reduce them to the point where the degree of secondary infection was much less severe. The use of disinfectants, e.g. mercuric chloride, was tested, but it was noted that the concentrations heavy enough to prevent contamination also prevented teliospore germination.

Both growing seedlings and individual leaves were used for inoculation with basidiospores by this method. From these, ten leaves

were found to bear a single pycnium; six were retained for inoculation with urediniospores. Four leaves, each with two widely spaced pycnia, were also retained. These four were divided into halves by cutting midway between the two pycnia. Each half was subsequently treated as if it were a single leaf, and will subsequently be referred to as such. Four of these leaves, plus the six whole leaves, were then inoculated with urediniospores. The other four, plus the remaining four whole leaves, were retained as controls.

(3) Reverse method - Flax seedlings, bearing uredinial pustules but no pycnia or aecia, were inoculated with basidiospores and incubated as in methods 1 and 2.

Subsequent periodic examinations for the appearance of pycnia and aecia were made.

Six selected leaves were inoculated by the single-basidiospore method, forty by the suspension method, and fourteen were retained as controls. The leaves inoculated by the suspension method were left on growing seedlings until examination for pycnia and aecia commenced; then they were detached for observation and subsequently floated on kinetin solutions.

The surface of each leaf that exhibited the presence of pycnia or aecia was further scrutinized under the microscope to ensure that no additional structures were present. Leaves on which the presence of aecia were detected, or suspected, were fixed in Carnoy's fluid and retained for sectioning; as were the controls. These leaves were imbedded in wax, and sectioned, then stained with aqueous safranin and counter-stained with alcoholic fast green in accordance with adaptations of

methods suggested by Johansen (23). The sections were made at thicknesses of 10, 15 or 20 microns. Each section was microscopically scrutinized to check observations made by surface examination.

Because the inoculated flax leaves usually also bore infections caused by other organisms, they were often badly distorted. This distortion made it difficult to obtain photographs which satisfactorily illustrated the observations made during microscopic examination. A secondary program was therefore initiated to obtain suitable photographs and for the purpose of general confirmation of the results of the primary trials. The flax variety Redwing was available from the greenhouse at the time, and since it, as is the variety Bison, is "universally susceptible," it was used in these trials. The required teliospore sori were also obtained from this source. A considerable number of plants already infected with uredinia were also inoculated with basidiospores by teliospore suspension, as in method 3.

Leaves and stem portions which bore uredinia and aecia, but only one or no pycnium, were selected from the treated seedlings. These were fixed in formalin-propiono-alcohol (5 parts formalin : 5 parts propionic acid : 90 parts 70% ethyl alcohol), then imbedded in wax and sectioned at a thickness of 15 microns. The sections were then stained with alcoholic safranin and counterstained with alcoholic fast green (23). In several instances, because some doubt existed concerning the identification of the infection stages under observation, a hand section of the unfixed leaf or stem through the area in question was made and examined immediately. Where this check revealed a condition warranting further examination, the remaining leaf or stem portions were retained for treatment in the above-mentioned manner.

RESULTS

The data from the principal trials are presented in Tables 1, 2 and 3.

The results obtained with the use of the single-basidiospore inoculation method proved to be rather limited. The tendency of basidiospores to adhere to dry surfaces made it necessary to use water as a transfer medium. In the presence of moisture, the spores germinated rapidly, often while still attached to the basidium. Moreover, since the basidiospores are rather small and hyaline, and the working magnification was, of necessity, relatively low, it was not possible to ascertain whether a spore had been deposited on the leaf in every instance.

Aecia appeared on two of the four leaves that had been inoculated with urediniospores but not on any of the controls. Examination of transverse sections of these leaves (including the controls) revealed only one pycnium on each.

Of the leaves treated by method 2, two of the ten leaves that had been inoculated with urediniospores bore a distinct aecium on each. Although aecial formation had apparently been initiated on most of the other eight leaves, this could not be verified because the leaves died before aecia developed sufficiently enough to allow positive identification. Only one pycnium was present on each of the two aforementioned leaves.

One of the control leaves produced an aecium, but an examination of the sectioned material revealed the presence of two closely spaced pycnia on the surface of the leaf immediately opposite. These pycnia were so closely spaced that they had appeared as one during surface

examination. Since no other stages had been permitted to infect the control leaves and were not observed to be present, it is most probable that these pycnia were of opposite mating types and had coalesced to form the aecium. No aecia appeared on any of the other control leaves, even though the presence of a single pycnium was verified on each. Since the control leaves, in general, survived longer than those that had been inoculated with urediniospores, they offered a longer period of time for aecia to form and be detected.

The use of method (3) (i.e. reversing the sequence of basidiospore and urediniospore inoculation) reduced the incidence of secondary infection. Because other organisms were not introduced as readily by urediniospore inoculations as by suspension of teliospore sori over the leaves, they had less time in which to infect the leaves. The results obtained by the use of this method therefore proved to be the most satisfactory. However, some leaf distortion, which tended to obscure several of the observations, still occurred.

Aecia were formed on two of the six leaves that had been inoculated with single basidiospores. No evidence of accompanying pycnia was found, however, even though transverse sections of the leaves were carefully examined.

On six of the forty leaves over which teliospore sori had been suspended, aecia were formed, again in the apparent absence of pycnia. Two other leaves each produced an aecium in the presence of a single pycnium. Many of the remaining leaves also produced aecia but had two or more pycnia present. These aecia could, however, be presumed to be the normal result of the coalescing of pycnia of different mating types, particularly since no aecia or pycnia appeared on any of the controls.

It was more difficult to induce germination of the teliospores in the secondary trials than it had been in the principal trials, therefore the production of basidiospores and the subsequent rate of inoculation of the flax leaves were considerably reduced. One advantage of this, however, was that the ratio of leaves bearing only one pycnium to those bearing a multiple number was increased.

It was hoped that the use of inoculation material from the greenhouse would reduce the severity of secondary infections. This did not occur and consequently the damage to the flax plants was comparable to that in the principal trials. It was this fact that made inspection of a large number of leaves necessary before a sufficient number of suitable photographs were obtained.

The observations made in the secondary trials were essentially the same as those made in the principal trials. In all cases, where a leaf or stem portion had been selected as bearing an aecium in the presence of a single pycnium, no other pycnia were detected. The relative number of aecia with no evidence of pycnia was considerably lower than in the principal trials. Usually an immature pycnium was observed, most often in conjunction with, and on the same side of the leaf as the aecium. None of these pycnia had reached a sporulating stage.

In contrast to the preceding observations, it was noted that leaves bearing no sign of uredinial infection seldom bore aecia, even though pycnia were present. Several of these leaves, on which clusters of sporulating pycnia occurred, were sectioned and examined microscopically, but no aecia were detected (fig. 3).

One observation, that had not been made in the principal trials, was that the appearance of aecia on uredinially infected leaves often

preceded that of pycnia. Normally pycnia appeared seven or eight days after basidiospore inoculation; however, in several instances they were detected on the fifth day after inoculation. On leaves not infected with uredinia, aecia did not appear until almost two weeks after inoculation with basidiospores, and invariably, some time after the appearance of pycnia. Most usually, on uredinially infected leaves, aecia and pycnia occurred more or less coincidentally.

Photographs illustrating the observations made in these trials are presented in plates 1 - 8.

Table I. Aecial formation on leaves treated by method 1: Single basidiospore inoculation followed by uredinial inoculation

Class	Total no. of leaves	No. with aecia	No. without aecia
Leaves inoculated with single basidiospores	20		
Leaves on which single pycnia occurred	8		
(a) Inoculated with urediniospores	4	2	2
(b) Controls	4	0	4

Table II. Aecial formation on leaves treated by method 2: Inoculation with basidiospores by suspension followed by uredinial inoculation of leaves bearing single pycnia

Class	Total no. of leaves	No. with aecia	No. without aecia
Leaves inoculated with basidiospores by suspension	Not counted		
Leaves obtained with single pycnia	17	-	-
(a) Inoculated with urediniospores	10	2	uncertain
(b) Controls	7	0	7

Table III. Aecial formation on leaves treated by method 3: Uredinial infection followed by inoculation with single basidiospores or by basidiospore suspension

Class	Total no. of leaves	No. with aecia & no pycnia	No. with aecia & pycnium	No. with aecia & 2 or more pycnia	No. with no aecia or pycnia
Leaves with uredinial infection	60				
I. Leaves inoculated with single basidiospores	6	2	0	0	4
II. Leaves inoculated with basidiospores by suspension	40	6	2	23	9
Controls	14	0	0	0	14

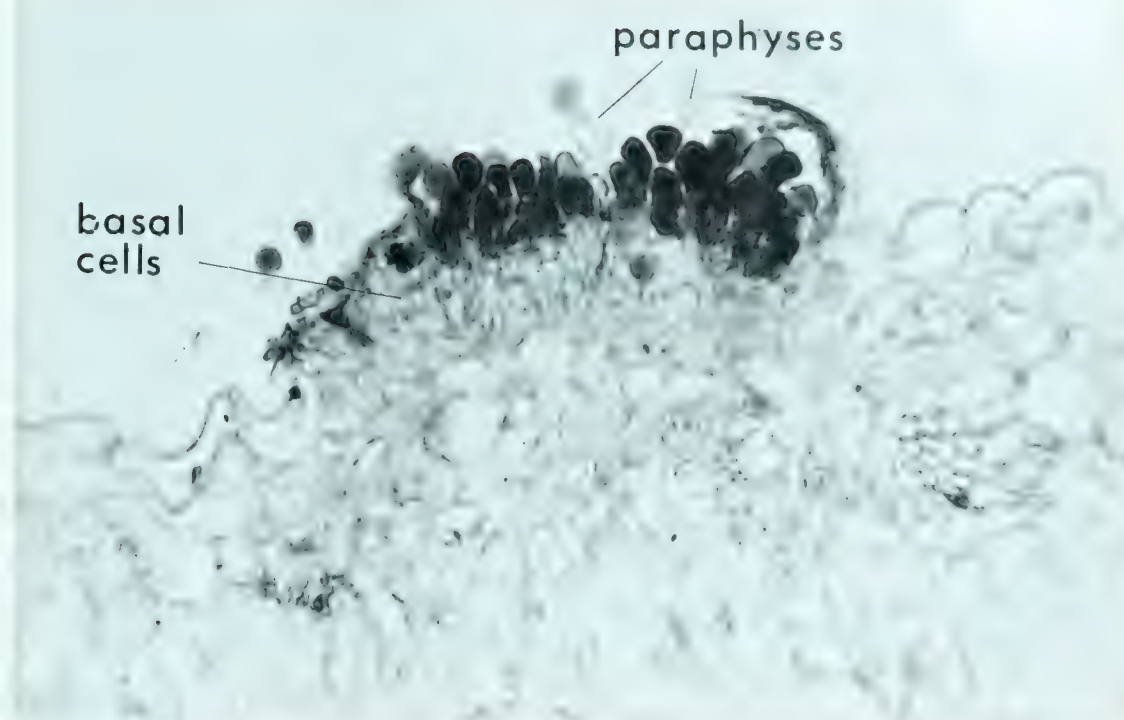


Fig. 1

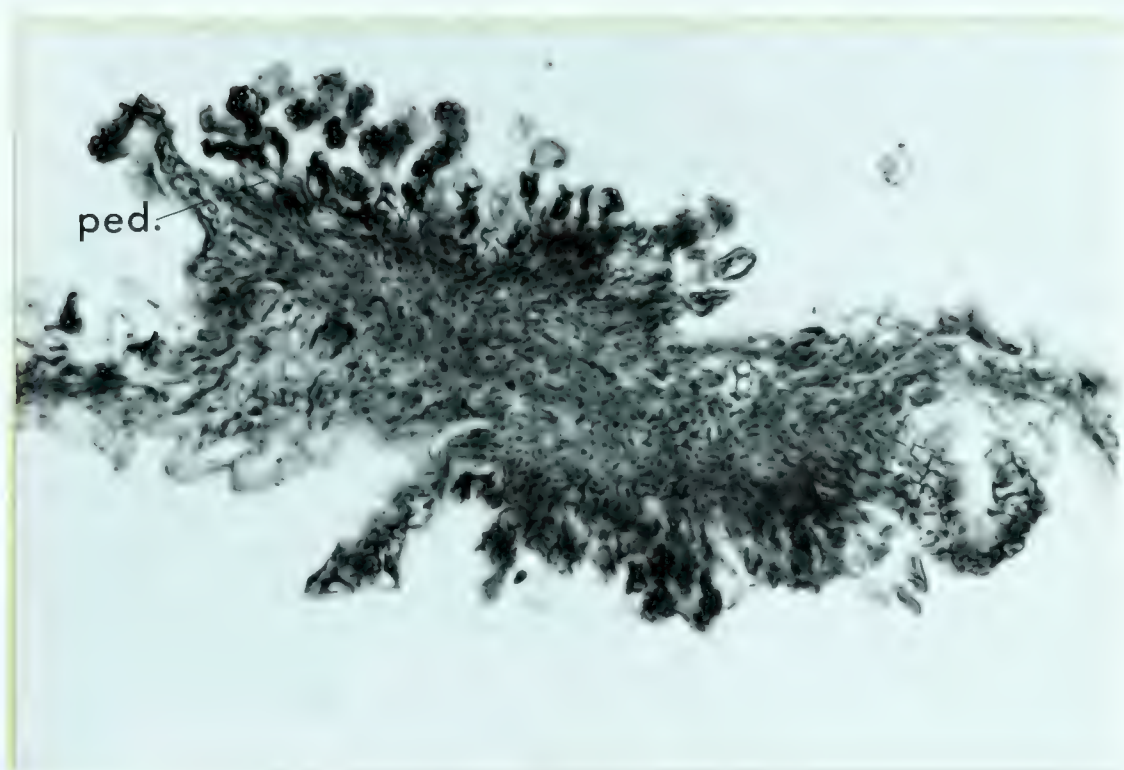


Fig. 2

Fig. 1. Transverse section through flax leaf, showing vertical section of young uredinial pustule. Approx. X250.

Fig. 2. Transverse section through flax leaf distorted by infection, showing vertical section of 2 mature uredinial pustules on opposite sides of the leaf. Approx. X250.

NOTE. All sections, unless otherwise indicated, were made at a thickness of 15 microns and photographed with direct illumination by white light.

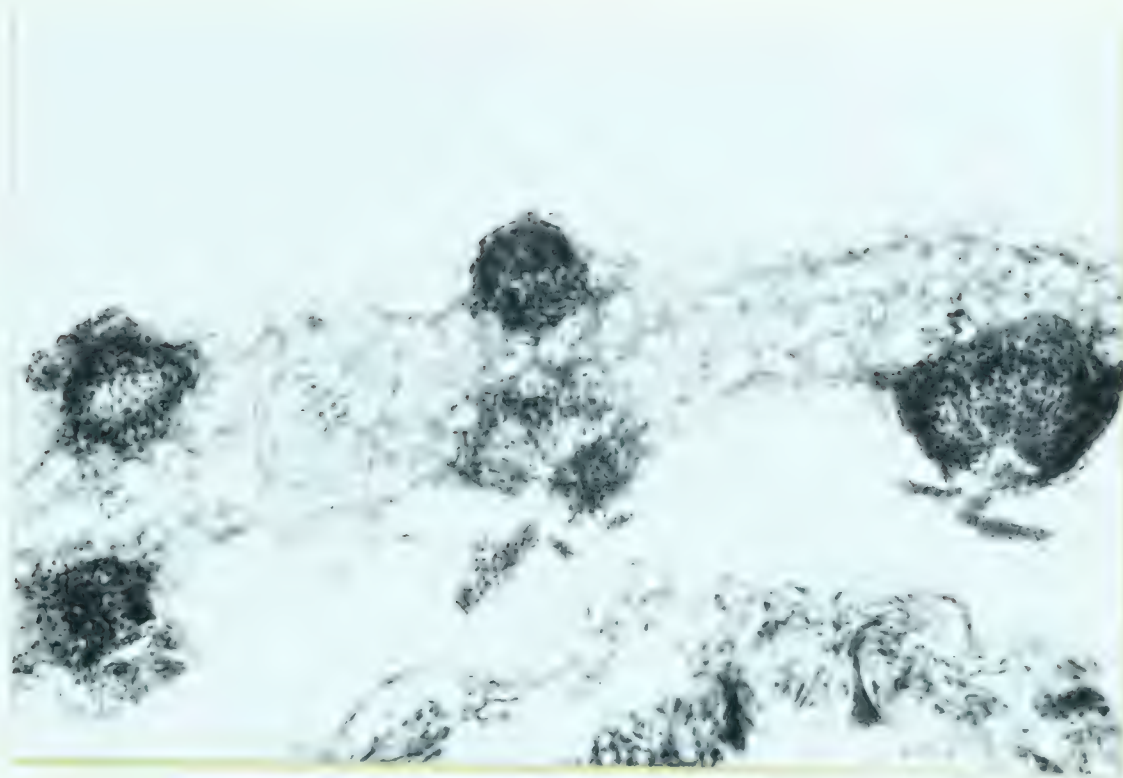


Fig. 3



Fig. 4

Fig. 3. Transverse section through flax leaf, showing cluster of five sporulating pycnia, with no evidence of an aecium. Approx. X100.

Fig. 4. Transverse section through flax stem, showing immature pycnia coalescing, with no evidence of aecia. Approx. X250.

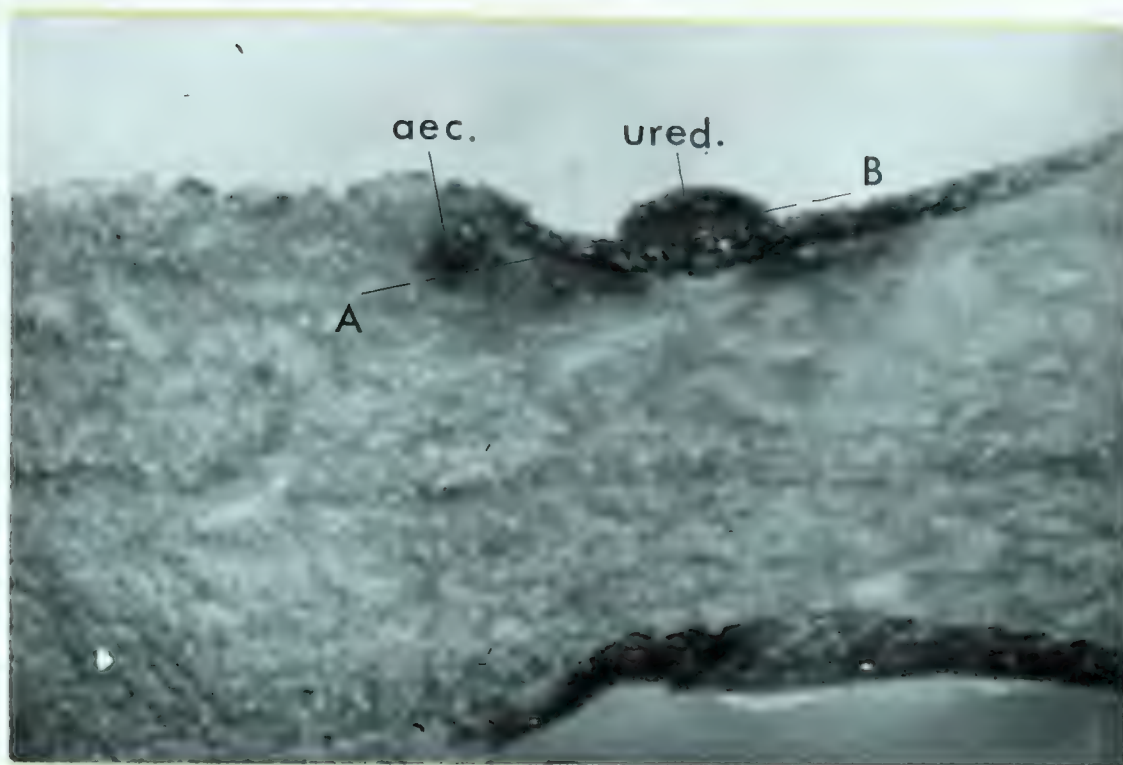


Fig. 5

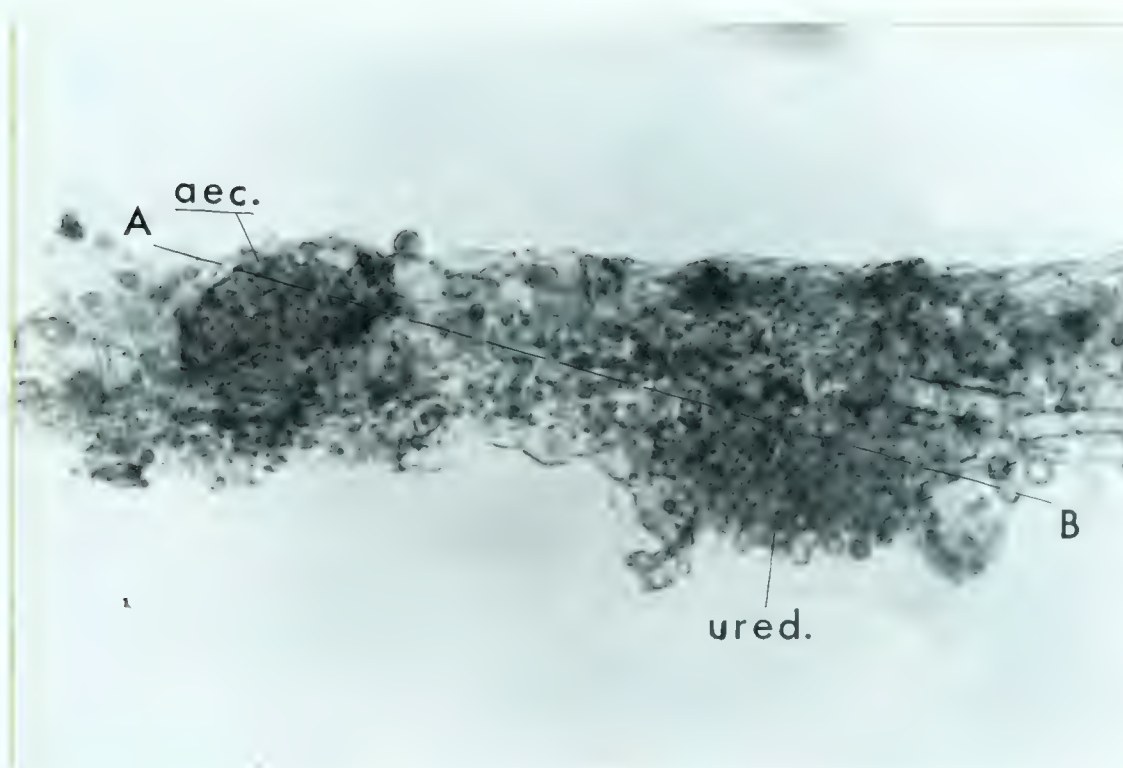


Fig. 6

Fig. 5. Surface of flax leaf, showing aecium and uredinium on opposite surfaces of the leaf, with no pycnia detectible. Lightly stained with fast green to increase contrast between leaf and pustules. Approx. X40.

Fig. 6. Hand cut section along A -- B of unfixed leaf in Fig. 5. Approx. X100.



Fig. 7



Fig. 8

Fig. 7. Transverse section through flax leaf, showing rudimentary pycnium in conjunction with an aecium, with an uredinium on the opposite side of the leaf. Photographed by monochromatic green light and phase contrast. Both aecium and uredinium have lost most of their spores. Approx. X400.

Fig. 8. Transverse section through edge of uredinially infected flax leaf, showing aecium with rudimentary pycnium. The pycnium is so closely conjoined to aecium it was not detected until sectioned and stained. Monochromatic green light and phase contrast. Approx. X350.

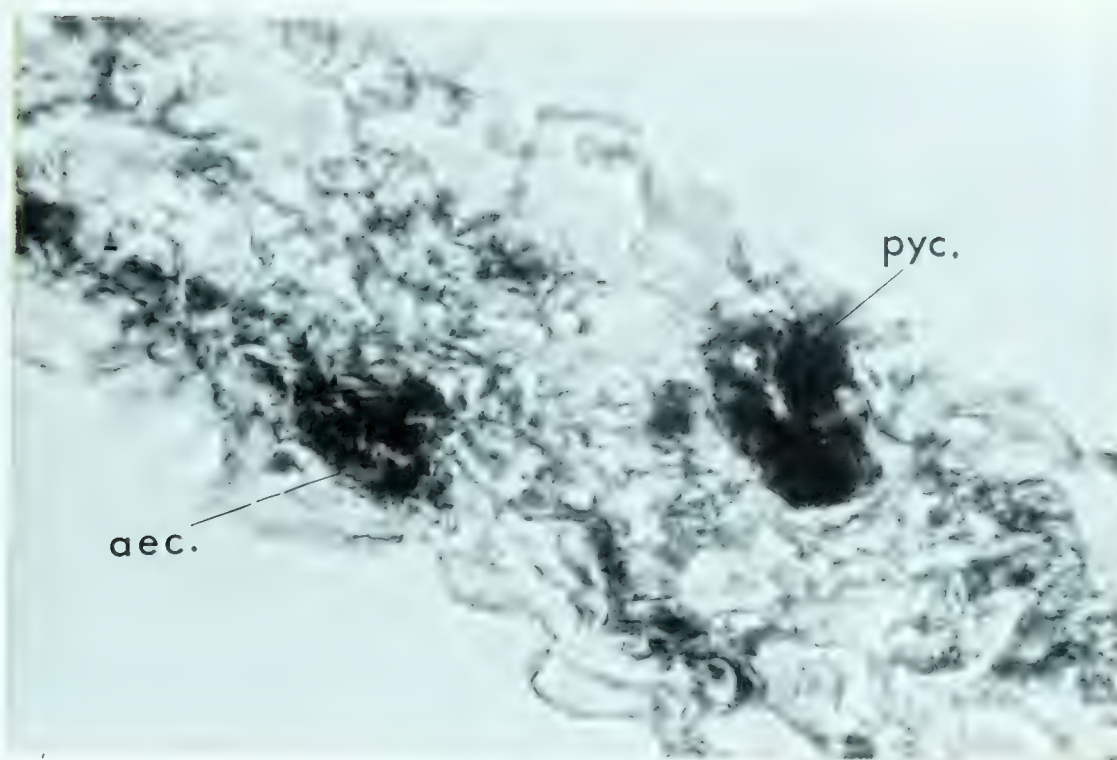


Fig. 9

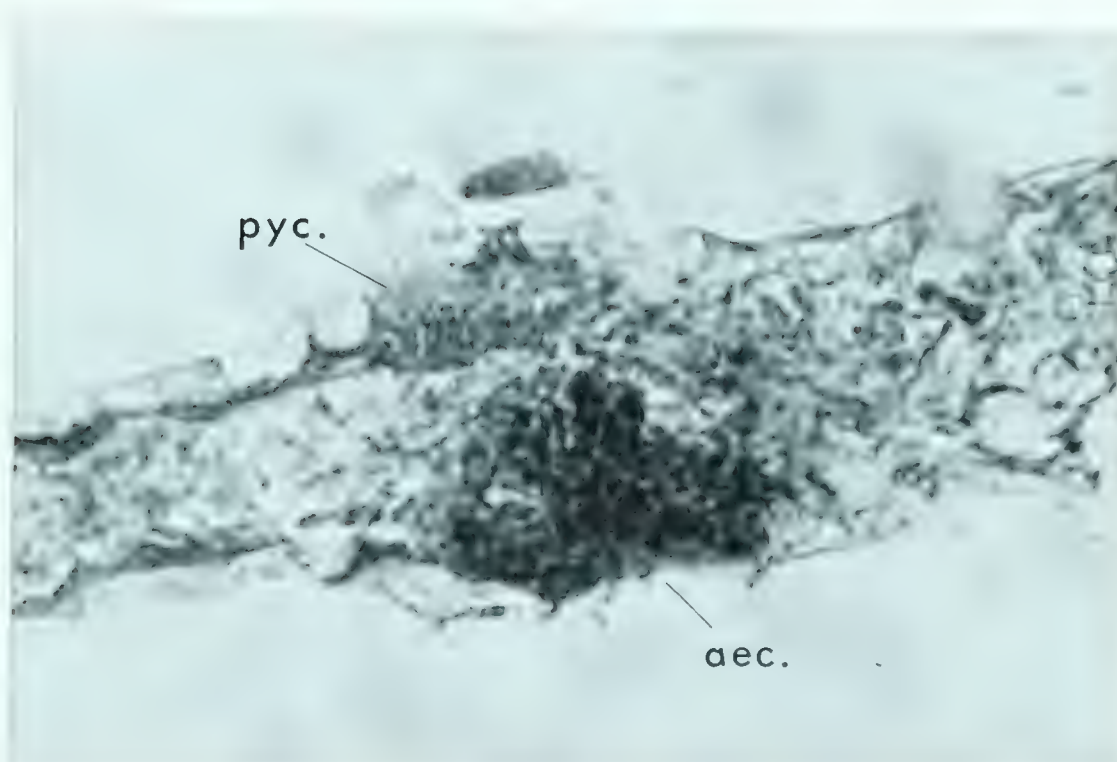


Fig. 10

Fig. 9. Transverse section through uredinially infected flax leaf, showing an aecium on the opposite side of the leaf from a pycnium. Both structures are newly initiated and excessively stained. Monochromatic green light and phase contrast. Approx. X350.

Fig. 10. Section similar to that in Fig. 9 but at different stages of maturity. The pycnium is at the point of sporulation; the aecium is slightly more developed than the one in Fig. 9. Monochromatic green light and phase contrast. Approx. X250.

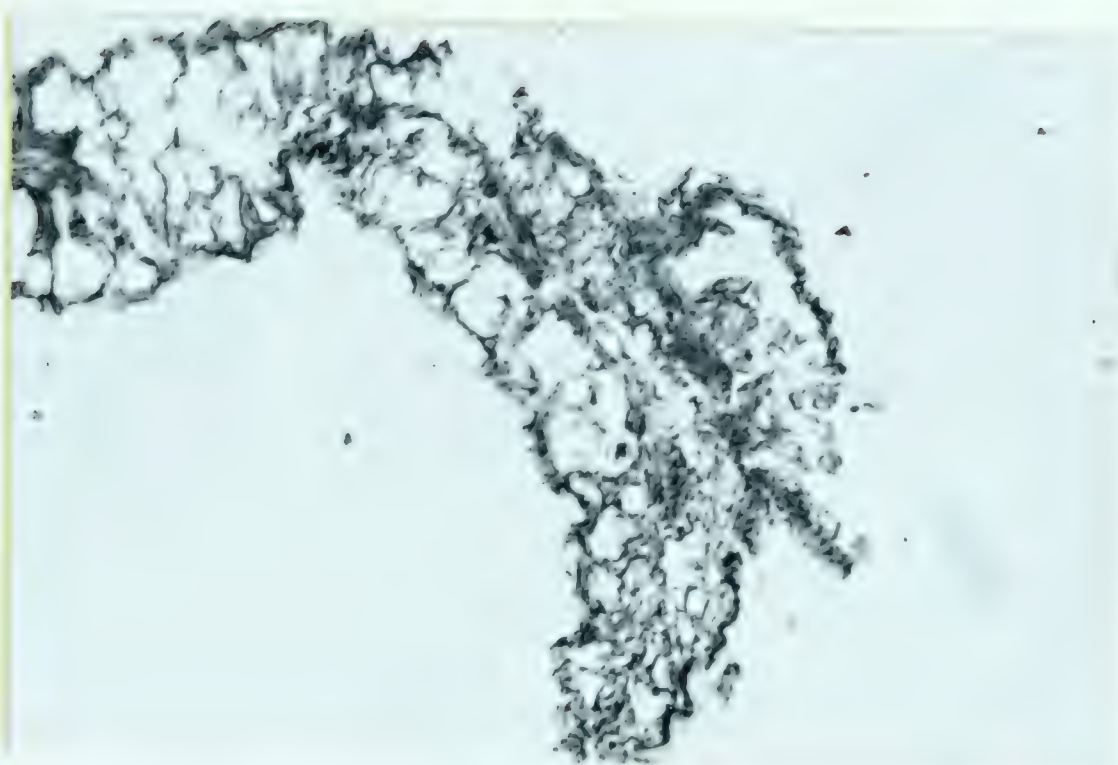


Fig. 11

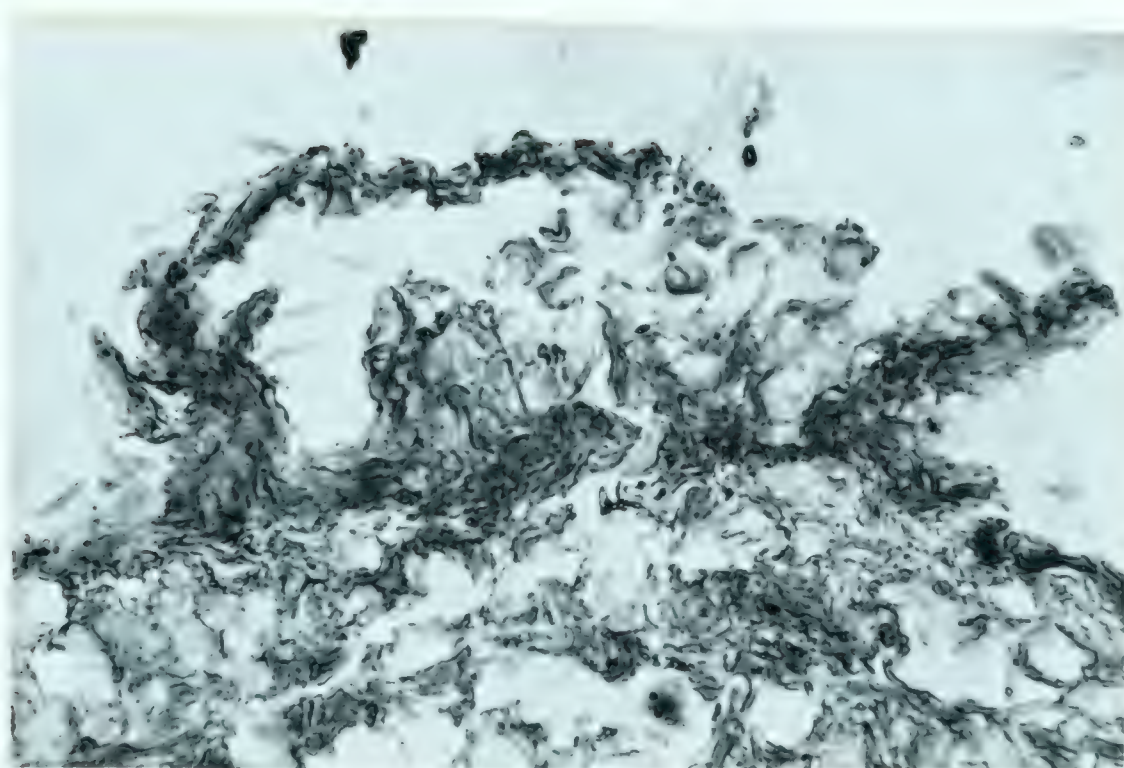


Fig. 12

Figs. 11 and 12. Transverse section through uredinially infected flax leaf, at different magnifications, showing an aecium with no detectible evidence of a pycnium. Approx. X200 and X350 respectively.

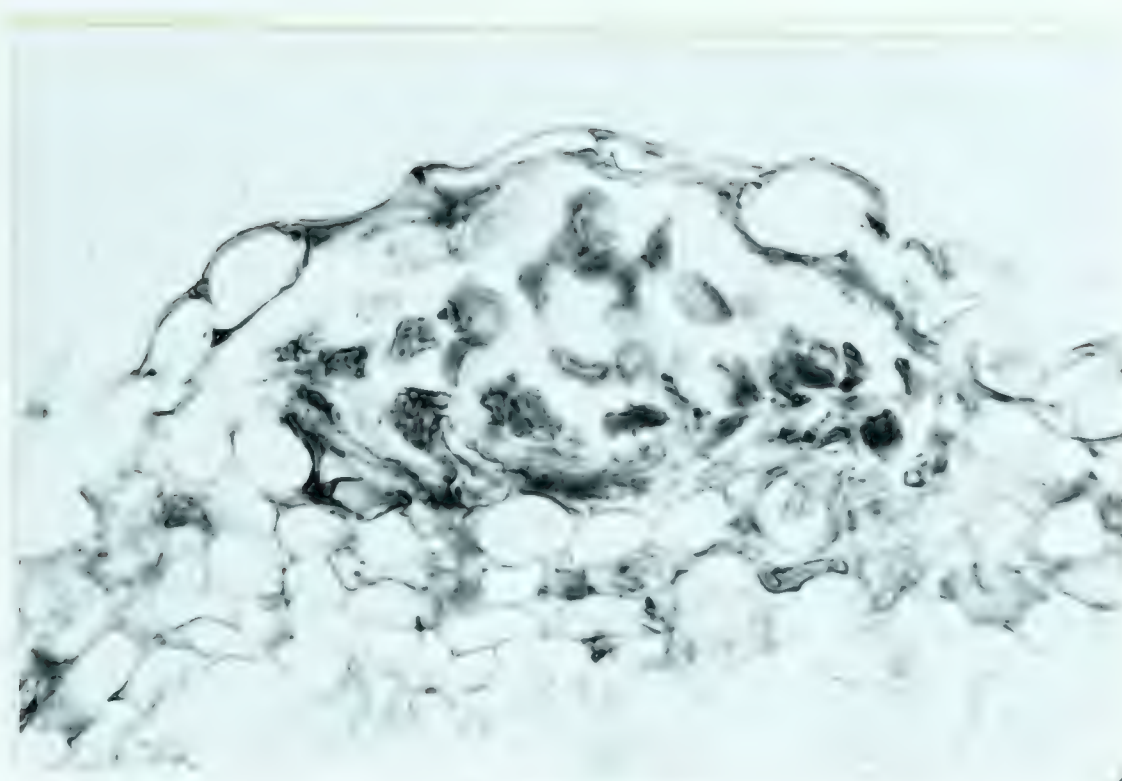


Fig. 13



Fig. 14

Fig. 13. Portion of a transverse section through stem of uredinially infected flax plant, showing an aecium with no detectable evidence of a pycnium. Approx. X350.

Fig. 14. Hand cut section of unfixed flax stem similar to the one in Fig. 13, showing basal cells which initiate aecial spore chains. Approx. X350.

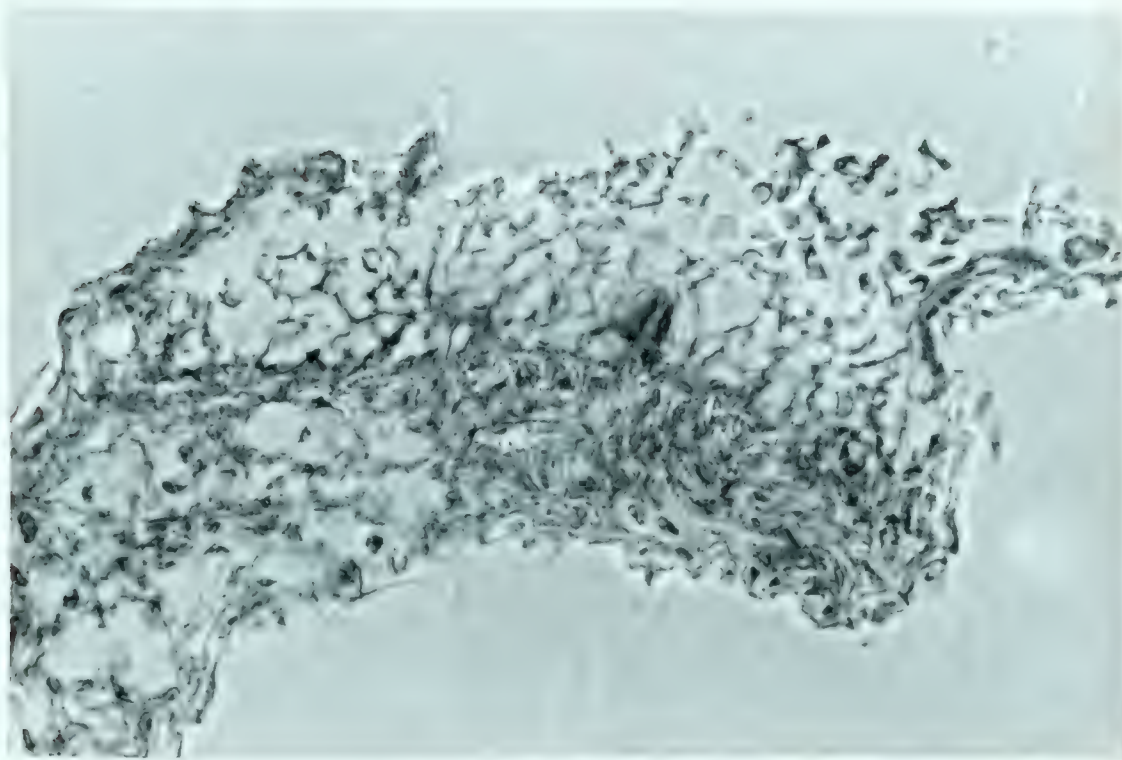


Fig. 15

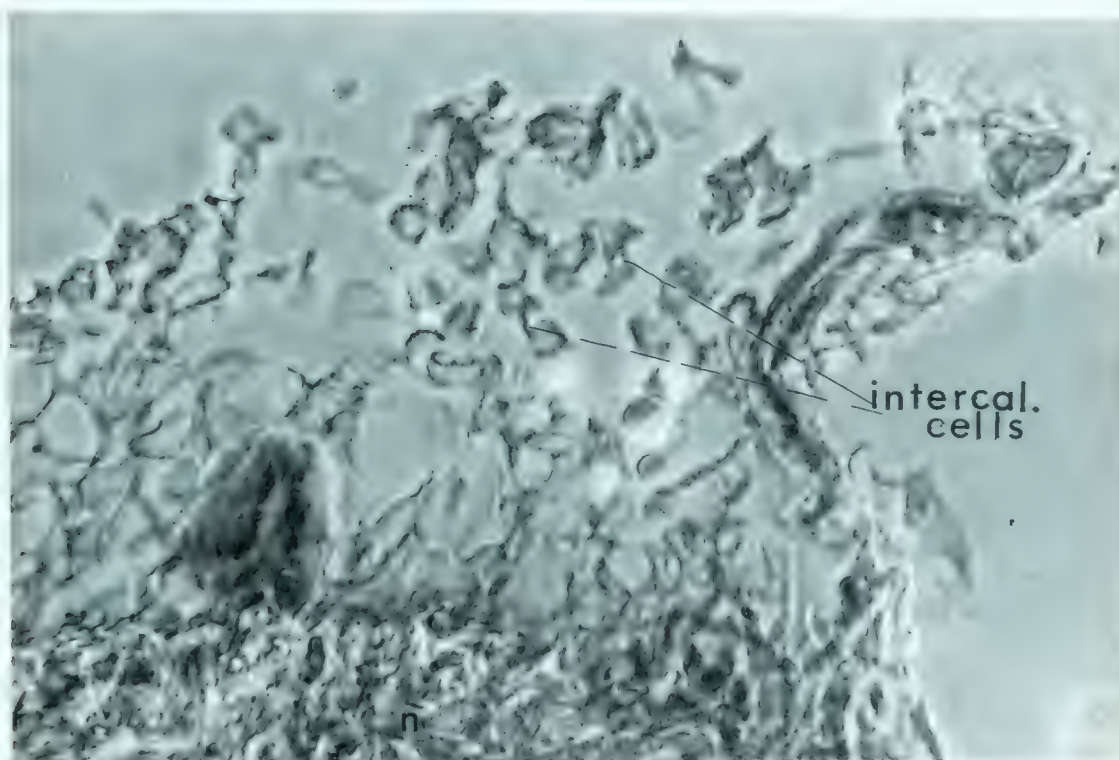


Fig. 16

Fig. 15. Transverse section through mature aecium on the edge of a flax leaf that had been infected with uredinia, showing remnants of aecial spore chains. Note the absence of any detectible pycnium. Approx. X250.

Fig. 16. Increased magnification of section in Fig. 15, revealing in greater detail the texture of spore walls and the structure of basal cells, also showing several degenerating intercalary cells. Both figures photographed by monochromatic green light and phase contrast. Approx. X350.

DISCUSSION

In M. lini, as in other species of rust, the formation of aecia is considered to result, normally, from the conjugation of pycnial nuclei of different (plus and minus) mating types. This is usually induced by the fertilization of a flexuous hypha (trichogyne) by a pycniospore (15), or by the fusion of pycnial hyphae (4, 14, 15).

In related species of rust (P. graminis and P. helianthi) diploidization has been shown to occur when diploid hyphae fuse with haploid hyphae (8, 13, 21). The present trials were conducted to determine whether this form of diploidization can occur in M. lini.

The results of these trials revealed that in all cases where infection by M. lini was restricted to the production of a single pycnium no aecia were formed. This is to be expected, since all spores and hyphae from any one pycnium are of one mating type only, either plus or minus (15), and no conjugation of such nuclei could occur. The introduction of a diploid stage, however, by inoculation with urediniospores, would introduce nuclei of both plus and minus types. With the fusion of an uredinial hypha and a pycnial hypha, one or the other of these nuclei could then conjugate with a pycnial nucleus, and, in consequence, diploidization would occur and an aecium would be formed.

In all cases, in the present trials, where uredinial and pycnial infections were present concurrently, several leaves did produce aecia. Since none of the controls (with one explainable exception) produced aecia, it could be concluded, therefore, that diploidization of pycnial nuclei had occurred.

The possibility that some form of auto-diploidization of the uredinial stage had occurred may be ruled out, because, in no case were aecia formed when only an uredinial infection was present. Although aecia appeared on eight leaves which bore uredinial infection, but no visible evidence of pycnia, these leaves should not be considered to have been infected with the diploid stage alone, since they had also been inoculated with basidiospores. This argument is supported by the observation that none of the fourteen control leaves in this trial produced aecia, even though they differed from the "treated" leaves only in that they had not been inoculated with basidiospores.

The fact that aecial production was less frequent among the leaves inoculated by the single-spore method than among those inoculated by the suspension method may be explained by inherent difficulties in technique. In the single-spore method it was not always possible to ascertain that a spore had been deposited on the leaf, therefore, it is quite probable that some of these leaves had not been effectively inoculated. Diploidization in such "uninoculated leaves" would not be expected to occur.

In the secondary trials, it had been observed, that on uredinially infected leaves which had been inoculated with basidiospores, aecia sometimes appeared simultaneously with, or prior to pycnia. This superficially indicates that aecial initiation coincided with, or preceded, pycnial initiation. But, since aecia appeared only after pycnia had been established on leaves free of uredinia, it is apparent that the presence of uredinia had been instrumental in inducing early aecial formation. According to Allen (4), pycnia normally appear eight days after the inoculation of flax leaves with basidiospores, and aecia appear

three or four days after pycnial fertilization. This was also observed to be the case during the present trials, although, by examining the leaves under magnification pycnia could be detected as early as six or seven days after basidiospore inoculation. It is evident, therefore, that aecia may be initiated up to four days after the pycnia and yet develop into visibly detectible structures before the pycnia. Most probably, then, a pycnial primordium, on coming in contact with an uredinial hypha, fuses with it; diploidization of a pycnial nucleus by an uredinial nucleus occurs; and the formation of an aecium is thus initiated. Since aecia develop more rapidly than pycnia, both would therefore become perceptible approximately at the same time.

There are two possible explanations for the production of aecia without the apparent presence of pycnia. It is possible that a rudimentary pycnium was actually formed but was not readily discernible. Alternatively, it may be that no pycnium had been formed, but that a basidiospore germ tube had fused directly with an uredinial hypha. In the first case, the fusion of an uredinial hypha with a hypha of a rudimentary pycnium may have arrested further development of the pycnium. In the second case, the fusion of a basidiospore germ tube with an uredinial hypha may have led to the direct formation of an aecium without the production of a pycnium. In the secondary trials, the frequent detection of a rudimentary pycnium in conjunction with an aecium where the pycnium had not been superficially evident, supports the postulation that fusion had occurred between uredinial and pycnial elements. However, since in several cases no pycnia had been detected, the possibility that fusion occurs between uredinial hyphae and basidiospore germ tubes cannot be excluded..

On the basis of the combined results of the present trials, there is little doubt that the formation of aecia had been induced by means other than the fertilization of pycnial trichogynes by pycniospores, or by the fusion of pycnial hyphae. The results also demonstrate that where a single pycnium was present on a flax leaf, the presence of uredinia was required before an aecium could be formed. Since, under these conditions, the formation of an aecium is indicative of the occurrence of diploidization, it can only be concluded that diploidization had in fact occurred, and that diploidization of a haploid stage by a diploid stage is inducible in the flax rust M. lini.

It is possible, that since diploidization may be induced artificially in flax rust, it also occurs in nature as a normal sequence of events. This could happen when wind- or insect-borne basidiospores alight on plants already infected with uredinia, or when urediniospores infect plants on which pycnia were also present. Because rust infections in different areas may be at different stages of the life cycle, it is also quite possible that plants bearing one stage of infection at one locale may, in addition, be infected by spores of a different stage from plants in another location, where a different race of rust is prevalent. This would tend to increase hybridization and to produce a greater variety of races than would occur with normal fertilization by pycniospores produced in the same area, and probably by the same race.

If the postulation that uredinial hyphae fuse directly with basidiospore germ tubes is correct, this would present research workers with a relatively simple method of following the path of nuclear migration. By labelling basidiospore nuclei with tritiated thymidine (H^3), carbon 14 or phosphorus 32 before inoculation, they would be

rendered readily identifiable by the use of autoradiography. Progressive examinations would consequently reveal the manner in which conjugation and diploidization occur.

Diploidization may also be used to practical advantage in the identification of rust races. Since the genes for resistance are dominant in flax, whereas those for virulence in rust are, with one possible exception, recessive, and since many rust races collected in nature are heterozygous, the potential virulence of these races is often masked when standard identification tests are performed (20). To determine the allelic condition of an unknown rust race requires separating these alleles and testing them individually within a suitable genetic background. This background may be provided by selecting a series of differential flax varieties and inoculating each with an appropriately matched rust race. The flax varieties should each be homozygous dominant at a different single resistance locus but homozygous recessive at all the other resistance loci, and the rust races should be correspondingly homozygous recessive at a different single virulence locus but homozygous dominant at all other virulence loci, e.g.

- (1) R^1R^1 , r^2r^2 , r^3r^3 , --- r^nr^n ; and v^1v^1 , v^2v^2 , v^3v^3 , --- v^nv^n ;
- (2) r^1r^1 , R^2R^2 , r^3r^3 , --- r^nr^n ; and V^1V^1 , v^2v^2 , v^3v^3 , --- v^nv^n ; etc.

In any of these flax-rust combinations, a change in the virulence loci to the heterozygous state would not affect the ability of the rust to infect its particular host unless the change occurred at a recessive locus. By inducing several diploidizations between each of these known races and the unknown race, and then testing the infectivity of the resulting aeciospores on their specific host, the type of alleles contributed by the unknown race could be determined. For example, if

only half of the aecia that developed from several diploidizations between one of the known races and the unknown race produced aeciospores capable of infecting the specific matched host, then the unknown race was heterozygous at that particular locus; if none of the aeciospores infected the host, then the unknown race was homozygous dominant at that locus; if all the aeciospores were infective, then the race was homozygous recessive.

The above-mentioned procedure for identifying rust races may be carried out without the use of diploidization. However, this would require cultivating both races through the teliospore and basidiospore stages to the pycnial stage. Also, to ensure that both alleles in question from the unknown race were represented, the infectivity of the aeciospores should be tested on a statistical basis. The use of diploidization, however, would permit maintaining one of the races at the uredinial stage, and would thus eliminate the often difficult and time-consuming task of getting the teliospores of different cultures to germinate at one and the same time. Furthermore, if the spores from a single basidium of the unknown race were used for inoculations in each set of diploidizations, the aeciospores from two of the resulting aecia would contain one type of allele donated by the unknown race, and those from the other two aecia would contain the other type of allele. It would be necessary, therefore, to test the aeciospores from only three aecia to determine their infectivity.

A refinement in the technique of single-basidiospore inoculation would permit the selection of basidiospores from any position on the basidium, thus making single spore analyses possible. Not only would this reduce the populations of host and pathogen required for race testing, but it would also provide a convenient means of obtaining additional information on linkage and segregation.

SUMMARY

The discovery that the "Buller phenomenon" (diploidization) occurred in the rust species P. graminis and P. helianthi motivated the present study, an investigation of the possibility of this event occurring in the related species M. lini.

The method that was used in the attempt to induce diploidization was to infect flax leaves concurrently with diploid and haploid stages of the rust. Three basic techniques were employed:

- (1) The establishment of individual pycnia with single-basidiospore inoculations, followed by inoculation with urediniospores.
- (2) Establishment of individual pycnia by suspending germinating teliospores over flax leaves, followed by uredinial inoculation.
- (3) Establishment of uredinial infections followed by single-basidiospore inoculations or by the suspension of germinating teliospores over flax leaves.

The control consisted of leaves infected with only the first type of inoculation in each of these methods. The criterion used to perceive the occurrence of diploidization was the formation of aecia.

Two of the four leaves treated by method 1 produced aecia but the controls did not. Two of the ten leaves treated by method 2 produced aecia which were readily detectible. Although most of the other treated leaves also appeared to have produced aecia, they died before this could

be verified. Only one of the control leaves produced an aecium, but since two pycnia were also noted to be in close proximity on this leaf, it was automatically disqualified as a control. Of the forty-six leaves treated by method 3 eight produced aecia without any evidence of pycnia, two produced aecia in the presence of a single pycnium and most of the others produced aecia when two or more pycnia were also present. None of the controls produced aecia. All results were verified by microscopic examination of leaf surfaces and transverse sections.

A secondary trial was also instituted for the purpose of obtaining more suitable photographs. The procedure was basically the same as in method 3, but on a more extensive scale. The observations made in this trial were essentially similar to those in the main trials and served to corroborate the findings made in these trials.

The appearance of aecia without the observed presence of pycnia was postulated to have occurred in either of two ways. Firstly, a rudimentary pycnium may actually have been present but was insufficiently mature to be discernible. Secondly, fusion between an uredinial hypha and a basidiospore germ tube may have occurred. Both explanations require that diploidization should occur.

The observations made in the secondary trial tended to support the former postulation, but did not exclude the possibility of the latter.

The results obtained in these trials establish that as in P. graminis and P. helianthi, diploidization is possible in M. lini. This fact opens up several possibilities for improving rust research techniques and for gaining information more efficiently. It may also be that diploidization, occurring under natural conditions, is a means by which new rust races originate.

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